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ANALYSIS OF IODINE-LABELLED GLUCAGON DERIVATIVES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY*

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SUMMARY

A reversed-phase high-performance liquid chromatographic system is presented for the separation of iodinated glucagon from the corresponding unlabelled and oxidized hormone, obtained by iodination of glucagon using the hydrogen perioxide–lactoperoxidase or the chloramine-T method. As the iodinated derivatives are well separated from the iodinated and oxidized as well as from the unlabelled glucagon molecules, this separation technique has been used both for optimizing the iodination procedures and for the rapid, efficient purification of mono- and diiodinated glucagon derivatives.

INTRODUCTION

Polypeptide hormones labelled with ^{125}I are widely used as tracers in radioimmunoassays and in studies of hormone–receptor interactions. To be able to draw conclusions from such studies, a precise knowledge of the chemical state of the modified polypeptide hormone is required. We have been investigating the interaction of glucagon with its hepatic cell surface receptor[1]. Quantitative analysis of the interaction of glucagon with its receptors and the deduction of certain binding models and mechanisms require a homogeneous tracer molecule that should be as similar as possible to the native hormone in both structure and biological activity, and free from contaminants that might interfere with quantitative binding studies.

Glucagon is a 29-amino acid polypeptide hormone with the sequence His–Ser–Gln–Gly–Thr–Phe–Thr–Ser–Asp–Tyr–Ser–Lys–Tyr–Leu–Asp–Ser–Arg–Arg–Ala–Gln–Asp–Phe–Val–Gln–Trp–Leu–Met–Asn–Thr. The two tyrosine residues at positions 10 and 13 are the targets of iodination reactions; the methionine residue at position 27 is susceptible to oxidation to the sulphoxide[2] during the iodination procedure, even when the labelling reactions are carried out under mild conditions and with extremely short reaction times. Iodination according to conventional methods using either lactoperoxidase in the presence of hydrogen peroxide[3] or chloramine-T[4] therefore results in mixtures of differently modified molecules, some of which might exhibit reduced receptor binding affinity and biological potency[5,6].

* Dedicated to Professor Dr. E. Buddecke on the occasion of this 60th birthday.

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In this paper we present a convenient technique for the analysis of reaction products obtained on iodination of glucagon. This technique employs reversed-phase high-performance liquid chromatography (HPLC) on a tetramethylsilyl (TMS) column and leads to the complete separation of unlabelled, I-labelled and I-labelled-Met^{27}-oxidized glucagon. It can easily be scaled up for preparative purposes.

EXPERIMENTAL

Apparatus

The analyses were performed on a DuPont HPLC system, which consisted of a gradient controller (Series 8800), a pump module (Model 870), a thermostatable column compartment, a variable-wavelength UV spectrophotometer and a Rheodyne sample injector (Model 7125) with a 50-μl sample loop. The system was coupled to a one-channel recorder (LKB 2210).

Separations were performed on a DuPont 250 × 4.6 mm I.D. Zorbax-TMS column. The column effluent was collected in an Isco Model 1850 fraction collector. Radioactivity was counted off line in a multi-crystal gamma counter (Berthold LB 2100).

Chemicals

Crystalline porcine glucagon was obtained from Novo (Copenhagen, Denmark). Carrier-free $Na^{125}I$ was purchased from Amersham-Buchler (Braunschweig, G.F.R.). Chloramine-T (N-chloro-*p*-methylbenzenesulphonamide), sodium hydrogen sulphite, sodium metabisulphite, hydrogen peroxide, acetonitrile, potassium dihydrogen orthophosphate and orthophosphoric acid were obtained from Merck (Darmstadt, G.F.R.) and lactoperoxidase from Boehringer (Mannheim, G.F.R.).

Methods

Iodination by the lactoperoxidase method. Amounts of 10, 100 or 1000 nmol of sodium iodide in 25 μl of water were mixed with 100 nmol of glucagon in 200 μl of 0.2 *M* phosphate buffer (pH 7.2), then 5 μl of lactoperoxidase (5 mg/ml) and 50 μl of hydrogen peroxide (0.5, 5 or 50 m*M*) were added with stirring. The reaction was stopped after 1 min by the addition of 50 μl of sodium hydrogen sulphite solution (5, 50 or 50 m*M*). For HPLC analysis 20 μl of the reaction mixture were used without further purification.

Radioiodination by the lactoperoxidase method. A 0.5-mCi amount of ^{125}I (5 μl of IMS30) was mixed with 20 μl of 0.5 *M* phosphate buffer (pH 7.2) and 20 μl of a solution of 0.1 m*M* glucagon in 0.01 *M* hydrochloric acid, then 5 μl of a 100 μg/ml solution of lactoperoxidase and 10 μl of 0.2 m*M* hydrogen peroxide were added with stirring. The reaction was terminated after 1 min by the addition of 10 μl of 1 m*M* sodium hydrogen sulphite solution. The radioactive iodination mixture was purified as described by Pohl[7]. The specific activity of the tracer was 50 μCi/μg.

Iodination by the chloramine-T method. Amounts of 10, 100 or 1000 nmol of sodium iodide in 25 μl of water were mixed with 100 nmol of glucagon in 200 μl or 0.2 *M* phosphate buffer (pH 7.2), then 100 μl of 22 m*M* chloramine-T were added with stirring. The reaction was stopped after 30 sec by the addition of 100 μl of 220 m*M* sodium metabisulphite solution. For HPLC analysis 20 μl of the reaction mixture were used.

Radioiodination by the chloramine-T method. A 3.5-μg amount of glucagon was iodinated with 1 mCi of $Na^{125}I$ as described by Pohl[7]. The specific radioactivity of the purified labelled glucagon was 150 μCi/μg.

HPLC of components of iodination mixtures. Native and modified glucagon were analysed by reversed-phase HPLC on a Zorbax-TMS column using a linear gradient from 21 to 30% of acetonitrile in 50 m*M* potassium dihydrogen orthophosphate buffer (pH 2.5), completed in 15 min, with a flow-rate of 1 ml/min and at 40°C. The column eluate was monitored at 220 nm. Fractions of 0.5 ml were collected with a fraction collector and tested for ^{125}I radioactivity with a gamma counter.

Enzymatic digestion of purified [mono-^{125}I]glucagon. HPLC-purified [mono-^{125}I]glucagon was lyophilized, neutralized and cleaved in 0.1 *M* ammonium hydrogen carbonate buffer (pH 8.0) with trypsin (50 μg/ml) for 20 h at ambient temperature.

HPLC of the tryptic peptide fragments. The glucagon fragments were analysed on a Zorbax-TMS column using a linear gradient from 2 to 24% of acetonitrile in 10 m*M* potassium dihydrogen orthophosphate buffer (pH 2.5), completed in 10 min, and an additional linear gradient from 24 to 30%, completed in 10 min, with a flow-rate of 1 ml/min and at 40°C. Fractions of 0.5 ml were collected and counted in a gamma counter.

RESULTS

HPLC of lactoperoxidase iodinated glucagon

Lactoperoxidase-mediated iodination results in a heterogeneous mixture of peptides, the composition of which depends mainly on the ratio of iodine to glucagon and on the length of the iodination procedure. An iodination reaction lasting for 1 min and with an iodine to glucagon ratio of 1:10 yields a mixture containing two main components (peaks C and D) which absorb UV light at 220 nm (Fig. 2a). Peak C has the same retention time as native glucagon (Fig. 1). The material corresponding to peak D coelutes with the radioactivity of [^{125}I]glucagon produced by the lactoperoxidase–hydrogen peroxide procedure with $Na^{125}I$ under otherwise identical conditions

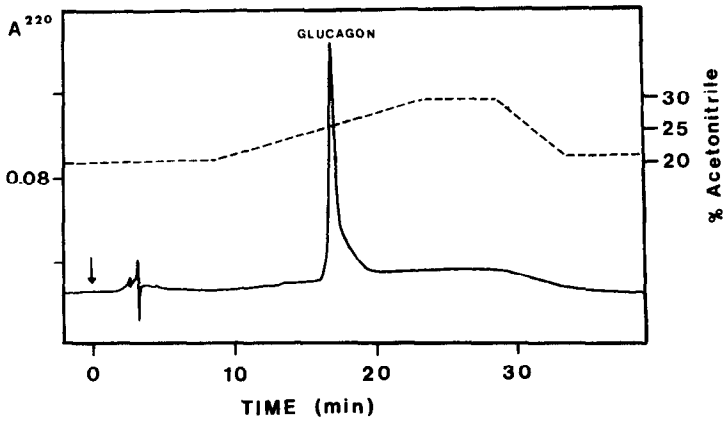

Fig. 1. Reversed-phase HPLC of glucagon. Column: Zorbax-TMS (25 cm × 4.6 mm I.D.). Eluent: A, 50 mM KH_2PO_4 (pH 2.5); B, 50 mM KH_2PO_4 (pH 2.5)–acetonitrile (40:60); linear gradient from 35% to 50% B in 15 min at 40°C; flow-rate, 1.0 ml/min.

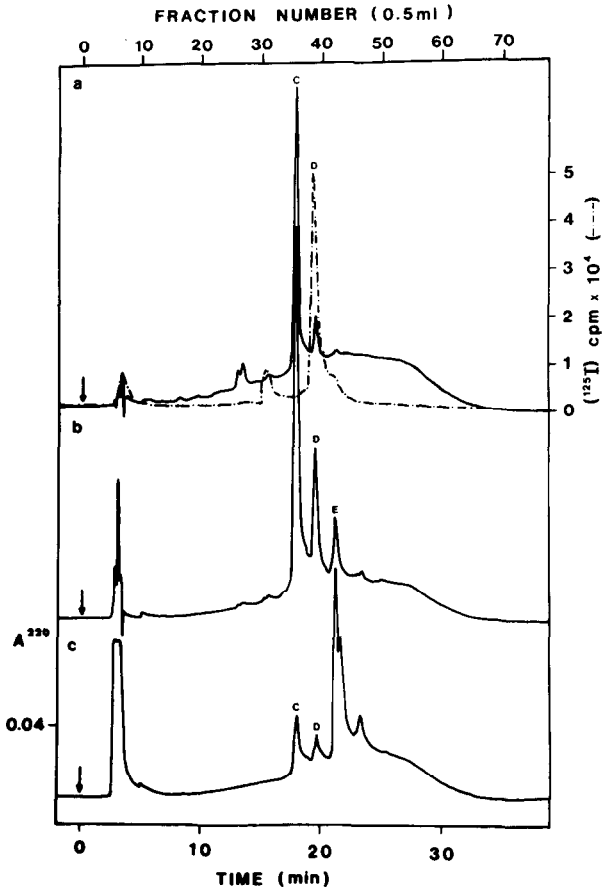

Fig. 2. HPLC elution profiles of lactoperoxidase-iodinated glucagon mixtures. Variation of the iodine content during the labeling procedure; iodine to glucagon ratio (a) 1:10, (b) 1:1 and (c) 10:1. Chromatographic conditions as in Fig. 1. The eluate was monitored for absorbance at 220 nm (——) and for the presence of ^{125}I radioactivity (– · – · –).

and is [mono-^{125}I]glucagon, *i.e.*, a mixture of Tyr^{10}- and Tyr^{13}-monoiodoglucagon, as will become apparent later in comparison with an analysis of glucagon iodinated by the chloramine-T method. An increase in the iodine to glucagon ratio to 1:1 during the labelling procedure results in characteristic changes in the HPLC elution profile indicating that, in addition to peaks C and D, polyiodinated glucagon (peak E in Fig. 2b) was produced. The shift to polyiodinated glucagon derivatives is even more pronounced after an iodination reaction performed with a 10-fold molar excess of iodine (Fig. 2c). The retention time of glucagon on the reversed-phase adsorbent increases with the degree of iodination, as described for other peptide hormones such as angiotensins[8], ACTH and MSH[9,10] and hexagastrin, Arg^8-vasopressin, oxytocin, Tyr^1-somatostatin and ACTH$^{1-39}$[11].

HPLC of chloramine-T iodinated glucagon

When the iodination of glucagon is performed by the chloramine-T method,

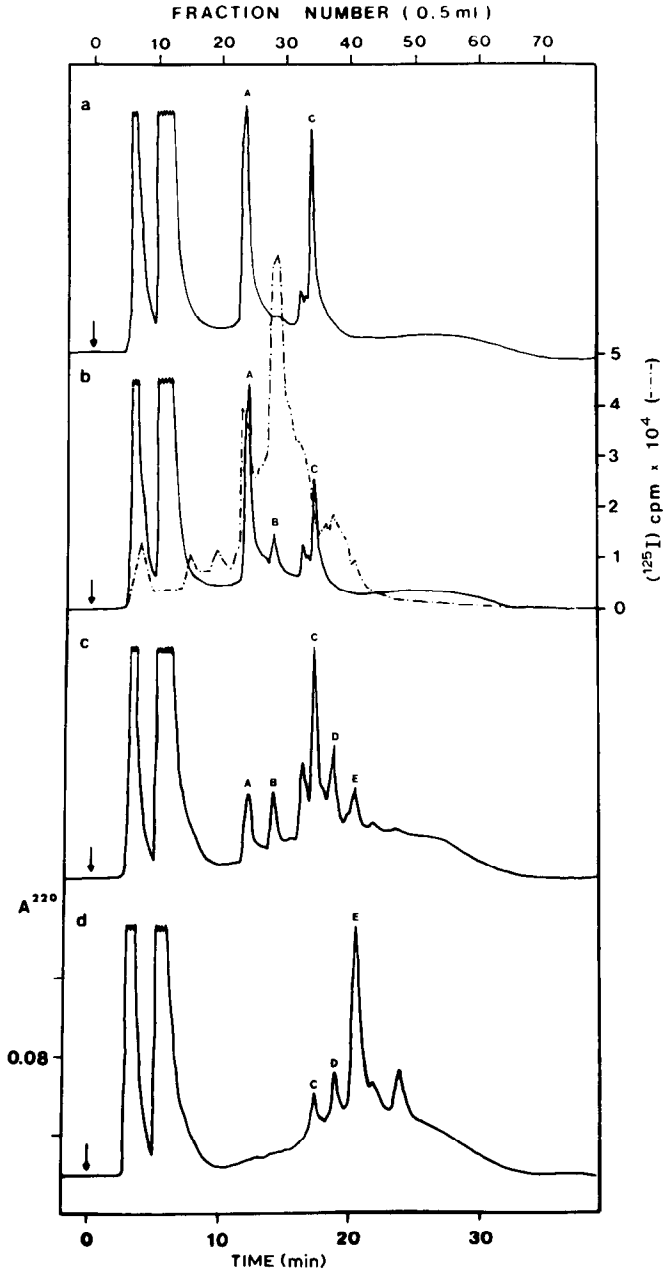

Fig. 3. HPLC elution profiles of (a) chloramine-T treated glucagon and (b–d) chloramine-T iodinated glucagon mixtures. The iodination reaction was performed (b) at a 1:10 ratio of iodine to glucagon, (c) at an equimolar ratio 1:1 and (d) with a 10-fold molar excess of iodine 10:1. The two large early eluting peaks correspond to $Na_2S_2O_5$ and chloramine-T. Chromatographic conditions as in Fig. 1. The eluate was monitored for absorbance at 220 nm (——) and ^{125}I for the presence of radioactivity (– · – · –).

the HPLC elution profile is different (Fig. 3). An iodination reaction time of 30 sec with a 10-fold molar excess of glucagon over iodine yields a mixture containing three major peaks (peaks A, B and C, Fig. 3b). Peak C represents unlabelled glucagon, peak A contains glucagon that is oxidized in the Met^{27} residue, as can be deduced from the control experiment, in which glucagon was treated with chloramine-T under conditions identical with those for iodination but in the absence of iodine (Fig. 3a). The material corresponding to peak B coelutes with the radioactivity of chloramine-T

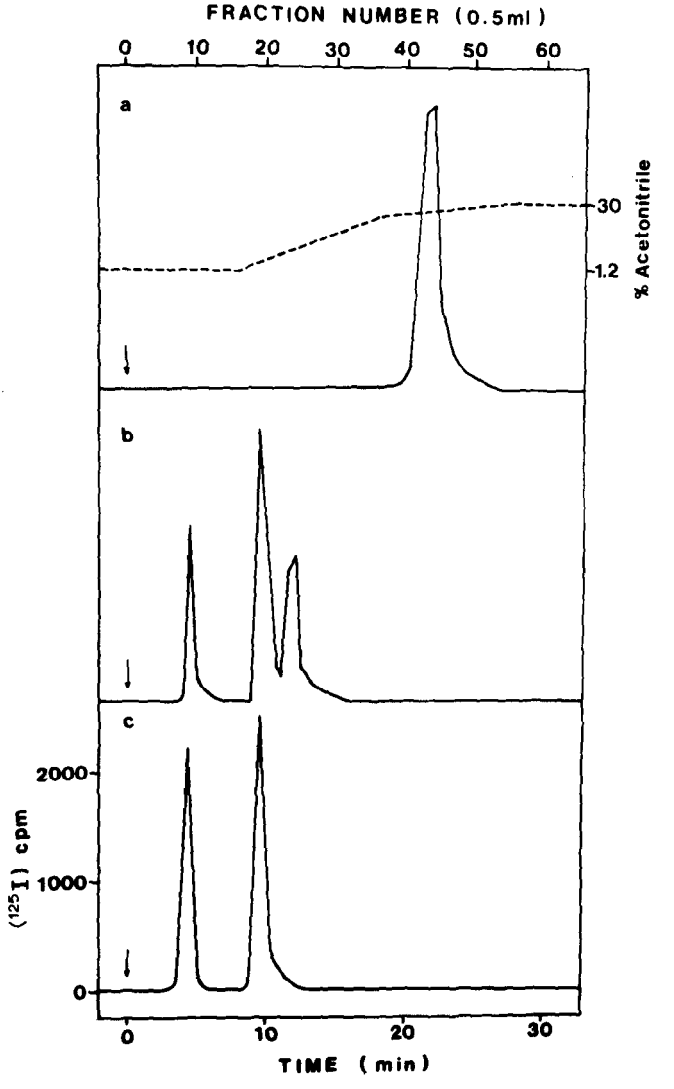

Fig. 4. HPLC separation of the peptidic fragments of [mono-^{125}I]glucagon obtained after digestion with trypsin. (a) HPLC-purified [mono-^{125}I]glucagon; tryptic digestion of [mono-^{125}I]glucagon after (b) 2 h and (c) after 20 h. Conditions for the cleavage are given under *Methods*. Eluent: A, 10 m*M* KH_2PO_4 (pH 2.5); B, 10 m*M* KH_2PO_4 (pH 2.5)–acetonitrile (40:60); linear gradient from 2% to 40% B in 10 min, 40% to 50% B in 10 min at 40°C; flow-rate, 1.0 ml/min. The eluate was monitored for ^{125}I radioactivity.

iodinated [^{125}I]glucagon and is monoiodinated oxidized glucagon. With increasing iodine concentration during the iodination reaction, the degree of incorporation of iodine into the tyrosine residues of the glucagon molecule increases. An equimolar ratio of iodine and glucagon increases the yield of monoiodinated glucagon, and in addition a new peak appears (peak E, Fig. 3c), which presumably is diiodoglucagon. A further increase in the iodine concentration during the iodination reaction by a factor of 10 results in an elution profile in which peaks at higher retention times appear, which presumably are due to polyiodinated glucagon (Fig. 3d). When the iodination reaction is performed with radioactive iodine (Fig. 3b), the major radioactive peak appears earlier than the radioactive peak in lactoperoxidase-labelled glucagon (Fig. 2a), the major radioactive peak being oxidized [mono-^{125}I]glucagon in Fig. 3b and [mono-^{125}I]glucagon in Fig. 2a.

Distribution of ^{125}I between Tyr^{10} and Tyr^{13}

In order to determine the location of iodine in the labelled glucagon, enzymatic cleavage of radioactively labelled monoiodoglucagon was carried out. The presumed [mono-^{125}I]glucagon (peak D, Fig. 2a) resulting from a 1-min iodination by the lactoperoxidase method with subsequent HPLC purification (Fig. 4a) was treated with trypsin, which cleaves glucagon behind Lys and Arg, at ambient temperature. After 2 h three radioactive fragments are separated by HPLC (Fig. 4b), one of which disappears on further incubation. After 20 h two radioactive peaks, each containing 50% of the applied radioactivity, are detectable. The radioactive fragments must be fragment 1–12 and fragment 13–17 originating from the two possible monoiodinated glucagon molecules. This result suggests that both tyrosine residues of the intact glucagon molecule are equally reactive towards electrophilic substitution by iodine, as [mono-^{125}I]glucagon (peak D) contains nearly equal amounts of Tyr^{10}- and Tyr^{13}-labelled glucagon.

CONCLUSIONS

The HPLC technique described here is a convenient method for the analysis of the product distribution from the iodination of glucagon. The lactoperoxidase and chloramine-T iodination methods produce several iodinated species that can be resolved on the TMS resin. The iodination conditions can be optimized owing to the possibility of analysing rapidly the reaction products that result from various iodination protocols. Further, this technique is suitable for the preparation of tracers of high purity and high specific activity. This now gives the opportunity of using a homogeneous tracer for the study of glucagon–receptor interactions.

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